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ABSTRACT

Tumor stem cell theory implies that causative lesions underlying human malignancies occur and are harbored in multi-potent progenitors with retained proliferative capacity and a prolonged lifespan. Transcriptional profiling of human breast cancers has identified five distinct subtypes of which the basal-epithelial subtype is most aggressive, correlates with poor prognosis, and lacks established molecular targets such as ERα, PR and Her2-overexpression. These tumors display a high degree of cellular heterogeneity suggesting that they may arise as the result of unregulated self-renewal in a multipotent cell. Clinically basal epithelial tumors are recognized by their negativity for ERα, PR and Her2, suggesting that the identification and validation of markers that definitively define this subtype will improve the diagnosis of these tumors. We present evidence that nestin, a previously-described neural progenitor marker is expressed in basal epithelia of the normal human mammary gland. Co-localization studies indicate two distinct populations of nestin-positive cells; one that expresses cytokeratin 14 and p63 and another that expresses desmin. Oncogenic transformation of a mammary progenitor cell culture model leads to increased expression of nestin. Immunohistochemical analysis of basal-epithelial breast tumors indicates robust expression of nestin, and CK14, punctate expression of p63, and low-to-undetectable levels of desmin expression. Nestin was not detected in other breast cancer sub-types, indicating selectivity for basal-epithelial breast tumors including those known to carry BRCA1 mutations. These studies coupled to the established role for p63 in the preservation of self-renewal suggest that basal breast tumors display biochemical features of mammary progenitors.

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Introduction

Little is known about the underlying causes of malignancy and this limits the ability of physicians and patients to make critical decisions regarding therapeutics as well as after-care strategies. The causative lesions of breast cancer are unknown as are the cells that harbor these lesions. Recent studies support a tumor stem cell theory, which holds that such lesions are happened in multi-potent progenitor with retained proliferative capacity and a prolonged lifespan. Throughout female reproductive life the epithelial portion of the mammary gland undergoes multiple periodic regenerative cycles characterized by cellular proliferation and terminal differentiation (1). During pregnancy there is a dramatic expansion of the epithelial compartment, followed by the acquisiton of physiological functions associated with lactation and extensive apoptosis and tissue remodeling during involution. In non-pregnant females, a less pronounced cycle of proliferation and cell death occurs with each menstrual cycle. Continuous regenerative cycling of the epithelial portion of the mammary gland depends upon a subset of Self-Renewing Basal Progenitors (SRBPs) that retain their proliferative capacity and resist termimal differentiation (2). These features confer a prolonged replicative lifespan indicating that such progenitor cells may be capable of accumulating mutations and harboring them from the protective effects of apoptosis. In addition, such mammary progenitor cells amy be the sites of breast cancer initiation, which is supported by studies in which mammary tumor stem cells were prospectively identified and shown to be uniquely tumorigenic and able to self-renew (3). These studies suggested that cancer initiation is a condition of unregulated or poorly regulated self-renewal and have focused attention upon potential of genetic pathways that regulate self-renewal. The aim of our proposal is to localize genes associated SRBPs' self-renewal or differentiation, which have prognostic value and may enhance the ability of clinicians to design therapeutic strategies that are specific to individual patients.

Transcriptional profiling of large cohorts of human breast cancers indicated that between 17 to 37 percent of tumor cases display a basal epithelial phenotype (4-7). These tumors are highly aggressive, poorly differentiated and lack molecular targets for endocrine or anti-Her2 therapy. The basal phenotype is associated with an early age of onset, and short times to

relapse and disease progression. A disproportional high number of these tumors are detected during normal screening mammography intervals (8), which may reflect their aggressive nature and may also suggest that they are more difficult to detect radiologically. For these reasons the breast cancers with a basal epithelial phenotype contribute disproportional to breast cancer mortality (9). Therefore, a greater understanding of the etiology of these tumors may help to identify selective markers and therapeutic targets that might improve detection diagnosis and treatment of basal epithelial breast cancer subtype. In addition, due to its basal epithelial phenotype, it is reasonable to suppose abundance of breast cancer SRBPs in such specific breast tumors, presenting a good model for studying genes events associates with SRBPs' self-renewal or differentiation.

There has been abundant evidence that the p53 family member TP63 plays a critical role in making decision to preserve or forfeit mammary progenitor cells' self-renewing capacity (10-13). The gene encoding TP63 utilizes proximal and distal promoters to produce Trans-Activating (TA-p63) and N-terminally deleted (△N-p63) isoforms (14). In adult mammary gland, abundant data reveal that expression status of TP63 reflects preservation or forfeiture self-renewing capacity (15). Mutations in TP63 have been shown to underlie a broad spectrum of syndromes, such as Limb-Mammary Syndrome that have in common defects in the establishment or cellular stasis of a variety of epithelial and apocrine structures (12, 16). These defects are believed to underlie a genetic program of non-regerative differentiation that ultimately leads to the depletion of progenitor pools. Targeted ablation of TP63 in the mouse resulted in profound failure of both embryonic and adult epithelial and apocrine structures (11, 13). Additionally, studies using pan-p63 antibody (4A4) indicate that TP-p63 predominateds in the basal epithelia of mammary gland, suggesting that TP-63 may play an important role in the preservation of mammary progenitors (17). This is further supported by studies indicating that TP-63 is a marker of progenitor cell population in corneal keratinocytes and that repression of TP-63 expression is correlated with the transition to a transient amplifying cell population and terminal population (18). To further identify the isoform of TP-63, TA-63 or △

N-p63 is essential for preserving progenitor cells pool within multiple epithelial structures, it is worthy generating a isoform specific antibodies of TP 63 facilitating further study the role of △N-p63 in progenitor cells biological behavior.

Nestin is an intermediate filament protein that is expressed in neural stem cells and other cells with regenerative potential (19). It has been shown to co-localize with p63 within a subset of the limbal epithelial of the cornea (20). Analysis of the differentiation of these cells to mature corneal keratinocytes indicates cells that express high level of p63 represent the self-renewing progenitor population (18). This suggests that nestin may also be expressed in self-renewing progenitors and is consistent with multiple findings indicating that nestin is a marker of neural progenitors (21, 22). Together with p63 expression and its role in preservation of progenitor cells in multiple epithelial structures, we are interested in observation of nestin distribution pattern in normal mammary gland and breast caner, especially subtype with basal epithelial phenotype. Furthermore, another subtype of breast cancer, BRCA-1 associated tumors have been shown to cluster with the basal epithelial phenotype (23). The distribution pattern of nestin in such cohort breast cancer was also evaluated to further investigate the correlation between nestin and breast progenitor marker, △N-p63 and its diagnostic value. Moreover, to discover nestin's co-localization with p63 and biological function in breast progenitor cells, two immortalized mammary epithelial cell lines, IMEC and MCF-10A are applied as the model for in vitro analysis.

Body

Materials and Methods

Tissue Samples: Normal human mammary gland samples derived from reduction mammoplasty were identified from archived samples within the Tissue and Tumor Bank at Dartmouth Hitchcock Medical Center (DHMC). For breast tumors representing diverse subtypes, the files of the Department of Pathology at Dartmouth-Hitchcock Medical Center were reviewed to identify formalin fixed paraffin embedded (FFPE) samples representing tumors that were either, ER-/PR-Her2- or ER-/PR-/Her2+ (by FISH) or ER+/PR+. Identified tumors were evaluated to ensure that sufficient tissue existed within the paraffin blocks. Identification and collection of tissues and tumors was conducted in strict adherence with regulations related to the protection of patient identity. BRCA1-associated tumors were identified and selected from archived material obtained through the Family Risk Assessment Program at the Fox Chase Cancer Center (Philadelphia, PA). These tumor tissue samples were from women that had undergone genetic testing through the Clinical Molecular Genetics Laboratory at FCCC and were found to be carriers of a deleterious BRCA1 mutation.

Generation of TA-p63 and △N-p63 specific antibody: A peptide encoding the TA-p63 specific transactivation domain in exon3 of p63 gene sequence (Gene bank No. AF_075428) downstream of TA promoter was applied for antigen to be injected into chick host. The antibody was extracted from eggs of antigen treated chicken. The TA p63 peptide amino acid sequence is CIRMQDSDLSDPMW. Similarly, a peptide encoding △N-p63 specific sequence in exon3' of p63 gene downstream of △N promoter was used as antigen to immunize rabbit host. The antiserum was extracted from serum of antigen treated rabbit. The △N-p63 amino acid sequence is MLYLENNAQTQFSE. The primary fraction of antibody was further purified with antigen peptide pre-binded affinity resin respectively. Enzyme Linked ImmunoSorbent Assays (ELISAs) was applied to screen out the most efficient antibody with the highest tier.

Immunohistochemistry: The basal phenotype of these tumors was confirmed by positive immounohistochemical staining for CK 5/6. Additionally, paraffin blocks representing tumors

of the Her2 subtype (ER-/PR-/Her2 positive by FISH) and tumors with a luminal epithelial phenotype (ER+/PR+) were identified for comparative analysis from 1999 to 2005. Additionally FFPE samples of normal human mammary gland derived from reduction mammoplasty were identified and used for analysis of marker expression in the normal mammary gland. Cases with sufficient paraffin-embedded material were selected for this study. Briefly, 4-µm thick series sections were cut from all tissue paraffin blocks containing representative tumor samples. Sections were applied to charged glass slides (Superfrost Plus) and dried. Sections were deparaffinized in xylene, rehydrated through a series of graded alcohol, then placed in 10mM citrate buffer (pH6.0, antigen unmasking solution, Vector Laboratories, Inc, Burlingame, CA) and submitted to antigen retrieval using microwave for 15 min. After heating, the slides were allowed to cool to room temperature and briefly washed with distilled water. Endogenous peroxidase was blocked with 0.3% hydrogen peroxide in distilled water for 10 min. Two different nestin antibodies were used for staining to confirm its specificity. Samples to be stained for nestin (1) were blocked in 5% donkey serum in 0.1% tritonX-100 in PBS. Samples to be stained with nestin (2), cytokeratin 5, 14, alpha-smooth muscle actin (alpha-SMA) and p63 were similarly blocked with horse serum. Samples to be stained for ∆-N-p63 were similarly blocked with goat serum. And samples to be stained with TA-p63 were blocked with 100% seafish serum. All blocking was done for 30 min at 37℃. Immnohistochemistry was performed using an avidin-biotin peroxidase system. The following primary antibodies were incubated for 45 min at 37℃: nestin (1) (1:50, clone C-20, Santa Cruz, Santa Cruz, CA, USA); nestin (2) (1:50, clone 10c2, Santa Cruz, Santa Cruz, CA, USA); desmin (1:50, Santa Cruz, Santa Cruz, CA, USA); pan-p63 (1:100, clone 4A4, BD Pharmingen, San Diego, CA, USA); cytokeratin 14 (1:100, clone LL002, Neomarkers, Fremont, CA, USA); cytokeratin 5 (1:25, clone XM26, Neomarkers, Fremont, CA, USA); alpha-SMA (1:500, Sigma-Alorich, St. Louis, MO, USA); TA-p63 (1:250); △N-p63 (1:150). Following washes in PBST, a biotinylated secondary antibody (Vector Laboratories, Inc, Burlingame, CA, USA) was applied for 30 min at 37℃. Detection of nestin (1) staining was

blocked in 5% donkey anti-goat IgG (1:200). Detection of nestin (2), pan-p63, cytokeratin 5, cytokeratin 14 and alpha-SMA was blocked with horse anti-mouse IgG (1:400). Detection of TA-p63 was blocked with goat anti-chicken IgG (1:200), and detection of △N-p63 was blocked with goat anti-rabbit IgG (1:200). Slides were incubated with strepavidin-peroxidase complex reagent (1:400, Vector Laboratories, Inc, Burlingame, CA, USA) for 30 min at 37℃ and developed with 3, 3-diaminobenzidine tetrahydrochloride (DAB) staining kit (Vector Laboratories, Inc, Burlingame, CA, USA). Mayer's haematoxylin was applied as a counter-stain. The slides were then dehydrated in a series of ethanol and mounted with Permount (Fisher, Fairlawn, NJ, USA).

Two-color Immmunofluorescence: The pretreatment and preparation of slides including deparaffinization, rehydration and antigen retrieval was identical to the protocols for immunohistochemistry. Blocking serum was applied for removing non-specific binding accordingly. Nestin (1) co-stained with nestin (2), pan-p63, cytokeratin 5 , cytokeratin 14 and alpha-SMA were blocked with 5% donkey serum. Then, the samples were incubated with two different primary antibodies together (nestin (1)+ nestin (2), pan-p63, cytokeratin 5, cytokeratin 14, alpha-SMA). Following washing with PBST, the slides were incubated with two different Alexa-fluor conjugated secondary antibodies (1:200, Invitrogen, Carlsbad, CA, USA) together. Nestin (1) co-stained with nestin (2), pan-p63, cytokeratin 5, cytokeratin 14 and alpha-SMA with donkey anti-goat-AlexFluor594 IgG and donkey anti-mouse-AlexFluo488. Slides were mounted with VECTASHIELD mounting medium with DAPI (Vector Laboratories, Burlingame, CA, USA) and imaged by fluorescence microscopy. The double staining protocol of △N-p63 plus cytokeratin 14 and alpha-SMA is applied similarly. The protocol of double staining of nestin (1) plus desmin, cytokeratin14 plus pan-p63 (4A4) is modified slightly due to identical host species of primary antibodies. The staining with primary antibody was conducted sequentially. The first primary antibody's staining was performed as regular immunohistochemistry protocol. The only difference was to use Avidin-AlexFluor488 (1:200, Invitrogen, Carlsbad, CA, USA) to replace the strepavidin-peroxidase complex reagent

followed by microwaving in 10 mM citrate buffer (pH6.0) for 15 min to remove non-specific binding. After such treatment, the slides were stained with the secondary primary antibody with typical immunofluoresence protocol and the AlexFluor 594 conjugated IgG accordingly.

Cell culture: IMECs and c-myc-transformed IMECs were cultured in DME/F12 supplemented with 5μg/ml insulin (InVitrogen), 10ng/ml EGF (Sigma), 0.5 μg/ml hydrocortisone (Sigma), 10mM Hepes-KOH (pH=7.3), 50 μg/ml BSA (Sigma), Pennicillin/Streptamycin, and 0.5 μg/ml puromycin (Sigma). Cells were fed every 48 hours and split 1-3 to 1-8 at confluence. The culture medium of MCF-10A cells was very similar but the only difference was 5μg/ml insulin and without hydrocortisone.

Northern Blotting: RNA was isolated from IMECs and c-myc-transformed IMECs using the RNAeasy system (Qiagen). Ten μg of RNA was loaded per well and northern blotting was conducted as previously described. Nestin mRNA was detected using an ~ 1kb EcoRI fragment derived from an IMAGE clone # 5493839 containing nestin cDNA sequences. Nothern blotting procedures were as previously described (24).

Semi-quantitative RT-PCR: RNA samples isolated from IMECs, c-myc-transformed IMECs, and MCF-10A cells were reverse transcripted to cDNA with Superscript III first-strand synthesis for RT-PCR (Invitrogen, Carlsbad, CA, USA). Two μg of RNA was used for reverse transcription each sample. The sequences of PCR primers are as follows: nestin (Gene bank No. NM_006617) forward 5'-ATC ACT GAA GTC TGC GGG ACA AGA-3'; reverse 5'-AAT TCT CCA GGT TCC ATG CTC CCA-3'; the PCR product size is 227bp. Δ-N-p63 (Gene bank No. AF_075428) forward 5'-ATG TTG TAC CTG GAA AAC-3'; reverse 5'-ATG GGG CAT GTC TTT GC-3'; the PCR product size is 350bp. GAPDH (Gene bank No. NM_002046) forward 5'-AAG GTC GGA GTC AAC GGA TTT GGT-3'; reverse 5'-AGT GAT GGC ATG GAC TGT GGT CAT-3'; the PCR product size is 510bp. The concentration of components in PCR reaction system are1×PCR buffer (10×), 2.5mM $MgCl_2$, 0.2mM dNTPs, 0.2μM primers, 4 units Taq polymerase (10 U/μl, Invitrogen, Carlsbad, CA, USA). 1.0 μl cDNA of total 25μl cDNA sample from 2μg RNA was used for each PCR reaction. The PCR conditions was as

follows: 95℃, 10min; then 95℃, 30s, 55℃,30s, 72℃, 1 min; total 35 cycles, 72℃, 10 min. 15μl PCR product was loaded to 1.5% SYBERGreen agrose gel for electrophoresis and observed with UV light.

Results

Nestin is expressed in the basal epithelial layer of the normal mammary gland.

Genetic analysis of TP63 in mice and humans indicates that it is required for the establishment (25) and preservation (11, 13) of SRBPs in multiple epithelial structures. Several studies have indicated that the dominant-negative isoform of p63, ΔN-p63 is highly expressed in the basal epithelia of the mammary gland and other epithelial structures and is required for the preservation of self-renewal (15, 17, 26). Studies of the limbal epithelia of the cornea indicate that TP63-α is expressed in the self-renewing population and this expression is repressed as cells forfeit their self-renewing capacity, enter a stage of transient amplification and achieve terminal differentiation (18). Other studies demonstrate co-localization of nestin and p63 in a subset of cells within the limbal epithelia (20). This study, coupled to abundant evidence that nestin is expressed in self-renewing populations (19) in the CNS and other sites in the body suggested that nestin may be expressed in the basal epithelia of the mammary gland. Formalin fixed paraffin-embedded (FFPE) samples of normal human mammary gland tissue were sectioned and subjected to immunohistochemical analysis of the expression of nestin, the basal epithelial marker, cytokeratin 14 (CK14) and the self-renewal marker p63. Two different primary antibodies of p63 were applied for immunohistochemistry staining. One is well-known 4A4 mouse monoclonal antibody commercially available. The disadvantage of such antibody is specificity due to its ability to recognize all TP63 isoforms in theory. Another antibody is the Δ-N-p63 antiserum generated in our lab, which could distinguish delta-N isoform in situ staining. Staining results indicate that nestin is robustly expressed in mammary ducts and lobules and that staining is evident in a layer of cells located between the luminal epithelia and the basement membrane (Figure 1A). To confirm that the detected signal was in fact nestin expression similar IHC was

conducted with a mouse monoclonal antibody directed against a separate epitope. Using this antibody we observed an identical pattern of expression (Figure 1B). In both mammary ducts and lobules nestin was detected in two morphologically distinct cell types. The first are a subset of columnar basal epithelia in which cytoplasmic nestin staining surrounds the nucleus. A second filamentous cell type is observed that is distributed along the periphery of the duct. It is important to note that regions within ducts and lobules are identifiable in which the filamentous nestin-positive cell type is distinct and physically separate from the nestin-positive columnar epithelia. This observation supports the assertion that nestin is expressed in two morphologically distinct subtypes. In addition to the analysis of nestin expression IHC was conducted to detect the basal epithelial marker CK14 (Figure 1C) and the progenitor cell renewal marker ∆-N-p63 (Figure 1D(4A4) and 1E(delta-N-specific)). Results (Figure 1C and 1D, 1E) indicate that CK14 is expressed in ductal basal epithelia and that p63 is present in the basal epithelia of both structures. These findings serve as a control for the characterization of nestin expression and support the assertion that nestin is expressed in the basal epithelia of the mammary gland. Interestingly, the distribution of another TP63 isform, TA-p63 is more universal and staining signal is much weaker that those of ∆N-p63. The TA-p63 positive epithelial cells are not only basal one, but also well-differentiated lumininal epithelial (Figure 1F). Such observations imply that different TP63 isoforms take over distinct biological function in maintenance of stability of mammary gland structure. ∆-N-p63 was very important for establishment and preservation of SRBPs, while TA-p63 might play a role in initiation of cellular differentiation.

Nestin independently co-localizes with mammary progenitor markers and myoepithelial markers.

To further confirm the specificity of nestin staining in breast, co-staining of two different nestin antibodies was used to verify identity of their staining pattern. Apparently, the two-color fluorescence overlapped totally showed that nestin signal is localized in subset basal epithelial cells and outer layer of filament structure (Figure 2I). The observation that nestin

was expressed in two morphologically distinct cell types in the basal epithelium of the human mammary gland coupled to multiple reports indicating that nestin expression is a common feature of regenerative cells within diverse tissues suggested that nestin may be co-expressed in SFRPs of the mammary gland with Δ-N-p63. Two-color immunofluourescence (IF) was used to determine if nestin was expressed in the same cells as the Δ-N-p63. Consistent with anti-nestin IHC, results indicate that in both mammary ducts and lobules, nestin is expressed in two morphologically distinct cell types. Additionally these studies indicate that nestin is co-expressed with p63 in a subset of mammary basal epithelia of both the duct (Figure 2A) and lobules (Figure 2B). In these cells the red-fluorescent signal associated with nestin expression is in close proximity to the green fluorescent nuclear signal associated with p63 and can be observed to surround p63-positive nuclei (Figure 2A inset), indicating that nestin and p63 are co-expressed in a subset of basal epithelia in both the ducts and lobules of the human mammary gland. To confirm these studies we sought to demonstrate that nestin is co-expressed with the basal epithelial marker cytokeratin 14 (CK14). Two color IF indicates that CK14 and p63 are co-expressed in cells within the columnar basal epithelia of the mammary gland (Figure 2C, 2D) and that nestin and CK14 are co-expressed in these cells (Figure 2E). We also noted that no ΔN-p63 or CK14 expression was detected in the nestin-positive filamentous cells that appear along the periphery of the ducts and lobules. The location and morphology of these cells suggested that they might represent myoepithelia or a myoepithelial precursor. Two well-recognized cellular myoepithelial markers, desmin and alpha-Smooth Muscle Actin (alpha-SMA) were used for investigate the phenotype of nestin positive cells localized outer layer of mammary gland structures. Other studies have noted that nestin is co-expressed with the striated muscle neurofilament protein, desmin in regenerating skeletal muscle (27). And there were also some evidence that TP63 could co-localize with alpha-SMA epithelial cells in normal breast duct (28-30). To determine if the filamentous nestin-positve cells were myoepithelial, two-color IF was conducted to determine if nestin was co-expressed with desmin in these

cells. Results (Figure 2F) indicate that nestin and desmin are co-localized in the filamentous myoepithelial cells. Additionally two-color IF was used to indicate that there is no overlap between desmin and ΔN-p63 (Figure 2G) or desmin and CK14 (Figure 2H). On the other hand, the co-staining of nestin, ΔN-p63 plus alpha-SMA revealed that there are some normal breast basal epithelial cells nestin or ΔN-p63 positive, while alpha-SMA stained negatively (Figure 2J, 2K, 2L). Taken together, these studies indicate that nestin is co-expressed in myoepithelia with desmin or alpha-SMA and also co-expressed separately with ΔN-p63 and CK14 in basal epithelia. They further suggest that nestin may play a role in regulating self-renewal or differentiation within ΔN-p63-positve SRBPs in the mammary gland.

Nestin transcript exists in breast immortalized basal epithelial cell line, IMEC and MCF-10A

The nestin distribution pattern of in situ Immunohistochemistry and immunofluoresence double staining had revealed that nestin protein exists in Δ-N-p63 and cytokeratin 14 positive normal breast basal epithelial cells. To further investigate biological function of nestin in regulation of self-renewal or differentiation and the correlation with Δ-N-p63 in basal epithelial cells, two immortalized breast epithelial cell line were applied to analyses the role of nestin played in such breast epithelial with progenitor behavior. The phenotype of MCF-10A is identical to that of IMEC, such as negative estrogen receptor expression, △N-p63 and cytokeratin positive, alpha-SMA negative, but the only difference is that immortalization of MCF-10A is result of chemical reagent rather than hTERT overexpression (31) . The semi-quantitative PCR was applied to investigate nestin response to retinoic acid treatment in MCF-10A and IMCE cells. In MCF-10A cells, Δ-N-p63 transcript level was down regulated by RA treatment, which was very similar to that in IMEC. Interestingly, although nestin transcript could be detected in both of breast immortalized basal epithelial cells, its response to RA treatment was not consistent with Δ-N-p63 totally. With retinoic acid treatment, nestin transcript could increase in 24, 48 hours and decrease in 96 hours in MCF-10A cells. On the contrary, without any treatment, nestin transcript level kept decreasing from 24 hours to 72

hours and could increase in 96 hours ultimately. In IMEC cells, the level of nestin transcript with RA treatment also showed same trend, but not so dramatically as in MCF-10A. Together with the existence of a retinoic acid response element (RARE) localized in upstream of nestin gene, the regulation mechanism of retinoic acid on nestin expression was more complicating and worthy of further investigation.

Oncogenic transformation of an Immortalized Mammary Epithelial Cell leads to Increased Expression of Nestin.

Prospective identification and isolation of mammary tumor stem cells indicate that they represent a very small proportion of a particular tumor (3). This implies that markers that are exclusively restricted to the self-renewing phenotype are likely to have limited clinical utility for the characterization of tumors. The finding that these prospectively identified tumor stem cells are uniquely tumorigenic and able to self-renew also suggested that cancer initiation may be a consequence of disregulated self-renewal resulting from mutations within normal stem cells. We hypothesized that the identification of genes that are upregulated in adult progenitors in response to oncogenic stimuli may help to identify useful markers of early carcinogenesis and may help to elucidate the mechanisms by which oncogenic transformation occurs.

Previously we reported the establishment of an Immortalized Mammary Epithelial Cell (IMEC) line via forced expression of the catalytic subunit of telomerase (hTERT) (15). In that study, we hypothesized that the lifespan of cells with a retained proliferative capacity maybe governed by telomeric erosion and that ectopic hTERT would enable these cells to elude replicative senescence. Characterization of multiple clonal isolates of the IMECs indicated a cytokeratin profile similar to that of basal epithelia and robust levels of ΔN-p63-α. These results coupled to multiple studies implicating ΔN-p63-α in preservation of mammary SRBPs indicated that IMECs displayed characteristics of mammary progenitors or other adult stem cells. An important question raised by the tumor stem cell model of carcinogenesis is whether tumor stem cells arise from adult stem cells. To address these questions IMECs

were stably transfected with c-myc or an empty vector control. Ectopic expression of c-myc in IMECs resulted in a transformed IMEC derivative that was anchorage independent (V. Cowling: personal communication). Using these IMEC derivatives we sought to determine if nestin expression was increased by oncogenic transformation. IMEC-EV (empty vector) and IMEC-myc were plated at 50% confluency and refed at Time=0. Total RNA was isolated at 0, 24, 48, 72 and 96 hours, and northern analysis of nestin expression was conducted to measure expression of nestin in IMEC-EV and IMEC-myc. Results (Figure 3) indicate that in IMEC-EV nestin mRNA is detectable at T=0 and and that levels decline by 24 hours and remain low through 96 hours. In the IMEC-myc derivative, expression of nestin mRNA at T=0 is comparable to that of IMEC-EV, however in this transformed derivative nestin mRNA levels increase steadily through 96 hours. This result indicates that c-myc-mediated transformation of IMECs caused increased expression of nestin. It further suggested that nestin might be a useful marker of progenitor-cell amplification in malignancies.

Breast tumors with a basal epithelial phenotype express nestin, ΔN-p63 and CK14.

The tumor stem cell theory of breast carcinogenesis implies that breast cancers may initiate within a population of cells capable of self-renewal (32). This may further suggest that tumors that are highly aggressive and poorly differentiated display more stem cell features. Large-scale gene expression profiling studies have lead to the identification of five breast cancer subtypes that can be correlated to clinical outcomes (4, 9). Of these five sub-types the basal epithelial subtype accounts for between 17 and 37% of the breast cancers surveyed in these large studies (5-7). These tumors express cytokeratin 5/6 and lack expression of ERα, PR and Her2 (ER-/PR-/Her2-). To determine if these tumors displayed features of mammary progenitors we selected tumors that were confirmed to be ERα-/PR-/Her2- and confirmed that these tumors expressed CK5/6 (33). Tumors meeting these criteria were then screened by immunohistochemistry for expression of nestin, CK14 and ΔN-p63. For each marker, positivity was defined as detectable expression within the tumor and not merely at the periphery of the tumor. For example, a ductal carcinoma in situ

(DCIS) in which the in tact basal epithelial layer stains positive but the cells within the core of the DCIS do not, would be scored as negative. Results indicate that in 14 of 16 basal tumors nestin expression was readily detectable. Figure 4A shows a representative sample of nestin staining of a basal epithelial tumor. Similarly 12 of 16 basal breast tumors were strongly positive for CK14 and 4 of 16 were weakly positive for CK14. Figure 4B shows a representative sample of CK14 staining of a basal epithelial tumor. Further analysis of p63 expression in these tumors indicated that 8 of 16 were positive and displayed a range of signal intensity from punctate (Figure 4C left panel) to intermediate (Figure 4C center panel) to uniform (Figure 4C right panel). Finally analysis of these tumors indicated that none showed any evidence of desmin expression (not shown). These studies indicate that basal epithelial breast tumors are positive for nestin, CK14 and p63 and negative for desmin. These studies suggest that basal breast tumors have a phenotype that is similar to mammary progenitor cells.

To determine if nestin was a selective marker of the basal breast cancer sub-type, we evaluated 16 tumors that were representative of the Her2 subtype (ER-/PR- and Her2 positive by FISH) and 16 tumors with a luminal epithelial phenotype (ER+/PR+). Under the same conditions in which nestin was detected in the basal tumors we failed to detect nestin in these other subtypes. Consistent with our analysis of the basal breast tumors, a postive signal for nestin was defined as immuno-detectable expression within the tumor. Nestin expression was detected at the periphery of ducts and in ductal carcinoma in situ (DCIS) but not in the tumor itself and these tumors were scored as negative. These studies (summarized in Table 1) while limited by low sample numbers indicate that nestin may be a selective marker of the basal breast cancer sub-type. Meanwhile, to further distinguish nestin expressed in basal epithelial cells from other myoepithelial marker staining, immunohistochemitry staining of alpha-SMA and desmin was also evaluated in same 16 triple negative breast cancer samples. Different from distribution pattern of nestin, desmin and alpha-SMA positive signals were localized in outer filament of tumor mass, but not inside tumor itself. Together with nestin

co-localization with Δ-N-p63 in normal breast, it implies that nestin might play a role as a progenitor marker and in regulation of self-renewal or differentiation in benign and malignant breast, not just as a filament structure protein.

Nestin expression is common in BRCA1-associated tumors.

Global transcriptional profiling of human breast cancers has indicated that that tumors that are associated with mutations in BRCA1 cluster within the basal breast cancer subtype (6). Given our previous result, which indicates that nestin is a selective marker of basal breast cancers, we sought to determine if expression of nestin was detectable in BRCA1-associated tumors. Immunohistochemical analysis of these tumors indicated robust nestin (Figure 4D) expression in 6 of 8 individual cases. Similar to the basal breast tumors studied above, CK14 (Figure 4E) and p63 (Figure 4F) expression were also detected in the BRCA1-associated tumors. These studies indicate that nestin expression is correlated with BRCA-1 associated tumors and is consistent with the finding that BRCA1-associated tumors are classified as a basal breast cancer. This data coupled to our studies indicating that in normal mammary gland nestin is co-expressed with markers of progenitor cell renewal may indicate that BRCA1 associated tumors display a progenitor-like phenotype. They further suggest that nestin may be a marker of BRCA1-associated tumors.

Discussion and future direction

We describe here the identification of the neural progenitor marker, nestin as a selective marker of the basal breast cancer subtype. We present evidence that nestin is expressed in two morphologically and biochemically distinct subtypes within the basal epithelia of the normal human mammary gland. In one of these cell types nestin is co-expressed with p63 suggesting that nestin may have a role in regulating self-renewal within mammary progenitors. Moreover, nestin transcript has been detected in two normal breast immortalized epithelial cells with basall phenotype, which further confirms the co-localization of nestin with progenitor cell marker, Δ-N-p63. We further report that oncogenic transformation of an IMEC cells leads to increased expression of nestin. Breast tumors representing the basal breast cancer

subtype (ER-/PR-/Her2- and CK5/6+) express robust levels of nestin and CK14 and display a punctate pattern of ΔN-p63 expression, suggesting that these tumors have a progenitor-like phenotype. This may be consistent with the aggressive nature and their poorly differentiated phenotype. Our studies also identify nestin as a potential target for molecular detection and diagnosis of breast cancers with a basal phenotype, including those with known BRCA1 mutations.

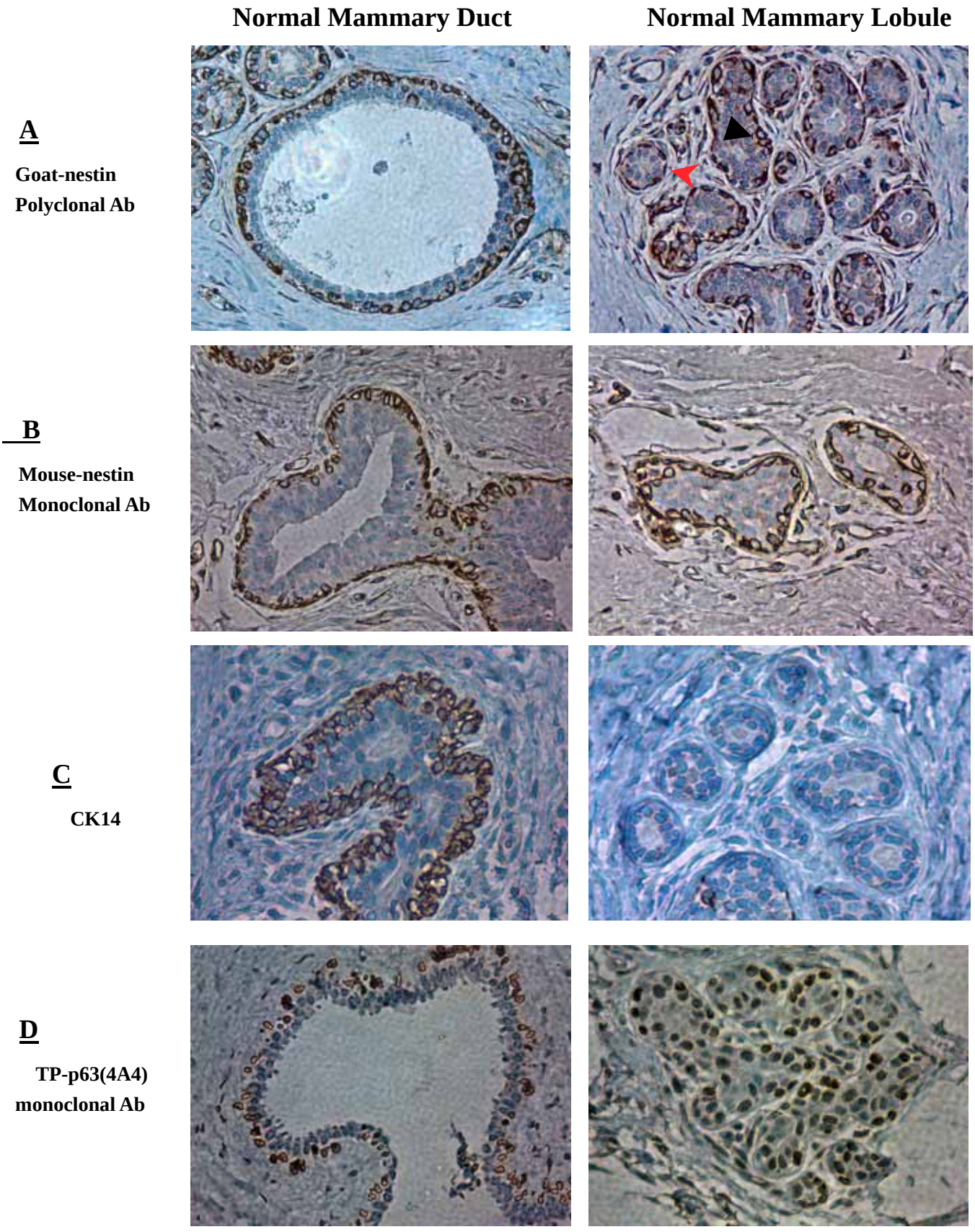
Genetic analysis of p63 indicates that it is required for the establishment (25) and preservation (11, 13) of epithelial progenitors. In our analysis of p63 expression in basal epithelial breast tumors eight of sixteen samples were observed to express p63 while none of the Her2-associated or luminal tumor types showed any detectable expression of p63. The expression pattern of p63 varied from punctate to uniform, which may suggest that the number of cells capable of self-renewal within a particular basal-epithelial tumor may vary. While it is unclear if p63 expression identifies tumor stem cells of the basal epithelial subtype, the observation of p63 expression in these tumors coupled to the finding that p63 is required for self-renewal may indicate that these cells have a retained self-renewing capacity. Further analysis of these cells will be required to determine if p63 expression underlies the self-renewing capacity of tumor stem cells in the basal epithelial subtype.

Our study indicates that nestin is expressed in the basal epithelia of the mammary gland and is a selective marker of the basal-epithelial breast cancer sub-type. While the precise function of nestin remains to be elucidated, several studies indicate that it may play a role in the regulation of mitosis within cells that have regenerative capacity (19, 34, 35). These findings coupled to the use of nestin as a marker of neural progenitors and the nestin promoter to selectively target neural progenitors suggests an important role for nestin in the regulation of some aspect of stem cell biology. The putative role of nestin in stem/progenitor cell regulation coupled to the finding that nestin expression is increased in c-myc transformed IMECs and in basal-epithelial breast tumors are potentially consistent with the idea that malignancies arise from self-renewing progenitors. Additionally the finding that nestin

expression was restricted to the most aggressive and least restricted breast tumor sub-type may suggest that the degree of progenitor-like features correlates with the aggressiveness and differentiation state of the tumors. This would further imply that the presence of nestin within a tumor might correlate with poor clinical prognosis. Additionally larger retrospective studies will be necessary to evaluate the prognostic significance of nestin. On the other hand, the reponse of nestin transcript to retinoic acid treatment is different from that of delta-N-p63 in MCF-10A and IMEC cells, which implicating the biological function of nestin in breast progenitor cell is more complicating as a well known progenitor cell marker in nervous system, and further analysis of the function of nestin in mammary progenitors may provide greater insight into self-renewal and differentiation processes. Except for nestin and Δ-N-p63, there are still some valuable progenitor markers in other tissue than breast, such as Scal-1 in haematopoietic cells, Mussashi in intestine, Bmi-1 in brain and Oct-4 in embryonic cells. The distribution of such progenitor markers in breast and breast cancers could be investigated by morphological methods such as immunohistochemisty staining, in situ hybridization. Further biological function analysis of these proteins could also provide promising insight into breast progenitor cell self-renewal regulation mechanism and localize more useful diagnostic marker for breast cancer.

Supporting data

Figure 1



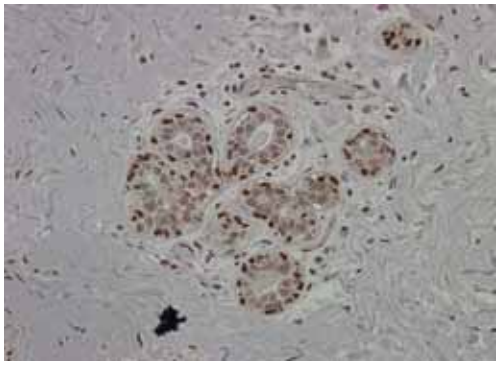
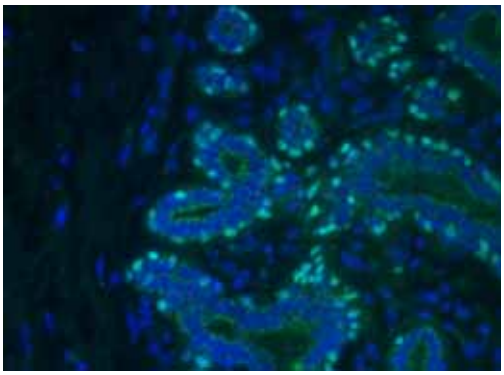
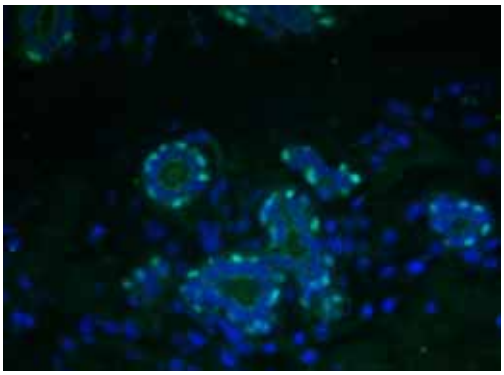
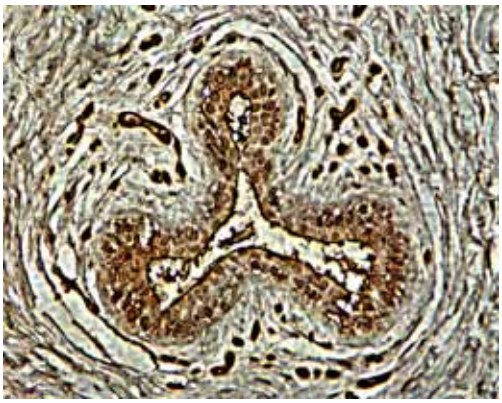
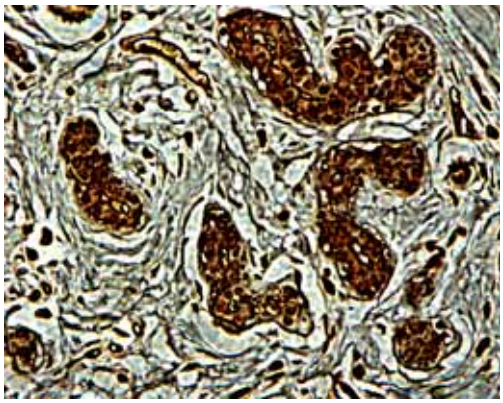
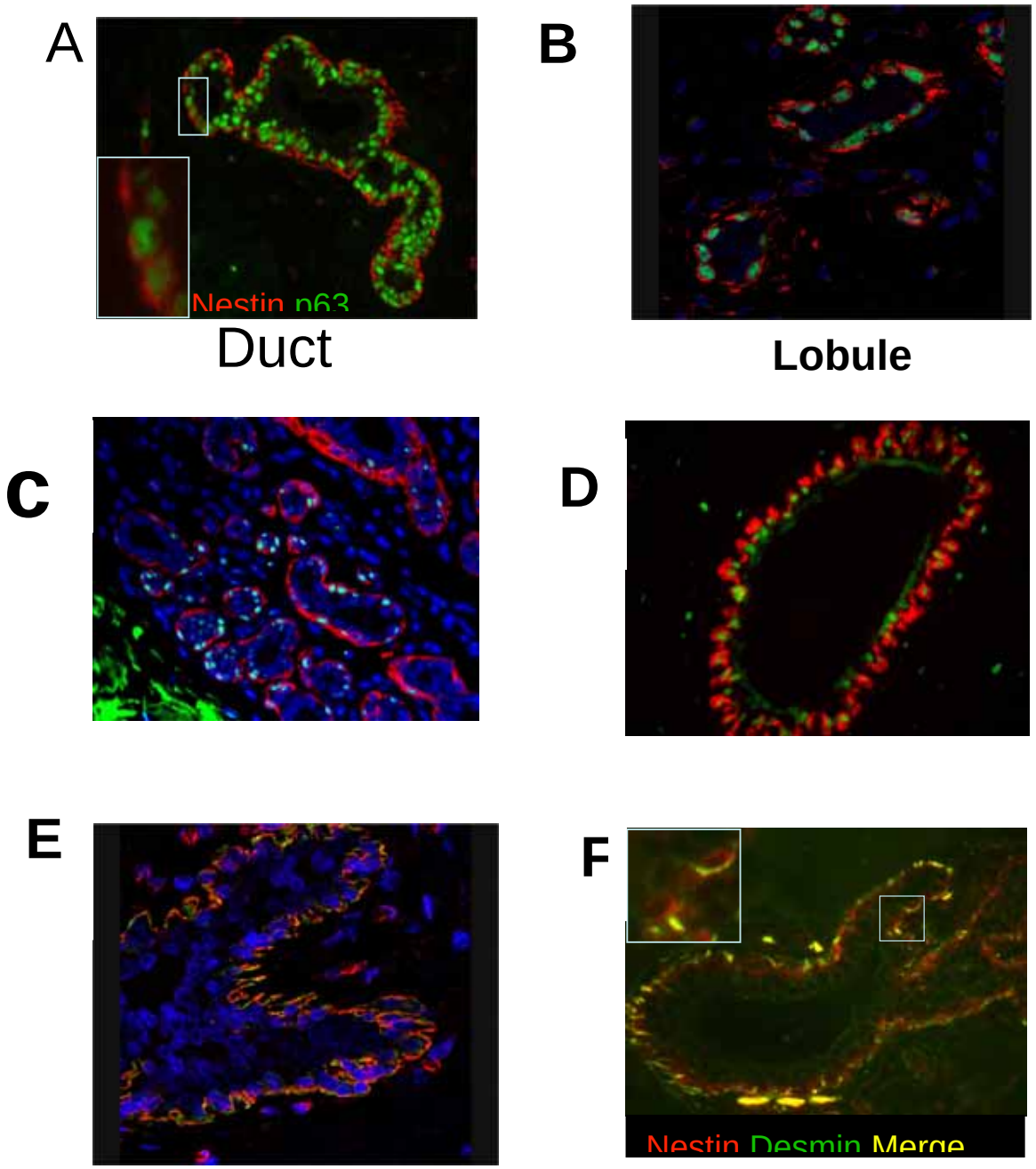
	Normal Mammary Duct	Normal Mammary Lobule
E **Rabbit delta-N-p63 Polyclonal Ab**		
F **Rabbit delta-N-p63 Polyclonal Ab**		
G **Chicken TA-p63 Polyclonal Ab**		

Figure 1: Nestin is expressed in two morphologically distinct extra-luminal mammary epithelial cell types. A. Formalin fixed paraffin embedded samples of normal human mammary gland, derived from reduction mammoplasty, were sectioned, applied to charged glass microscope slides, and subjected to immunohistochemical analysis for expression of nestin. Using a goat anti-nestin polyclonal antibody, staining of both ducts and lobules was observed in a layer of cells that is one cell removed from the luminal epithelial. Two specific cell morphologies were observed; columnar (indicated by black arrows) and filamentous (indicated by red arrows). Sections were counterstained with hematoxylin. B. To confirm the specificity of the goat anti-nestin monoclonal, similar analyses were conducted with a mouse anti-nestin monoclonal. Similar patterns were observed, confirming that the staining detected was due to the presence of nestin. Sections were counterstained with hematoxylin. C. Staining with the basal epithelial marker, Cytokeratin 14 (CK14) was done using a mouse anti-human CK14 monoclonal Ab. Staining was detected in all ducts but only rarely in mammary lobules. The right panel shows a representative section of mammary lobule in which no CK14 staining is evident. Sections were counterstained with hematoxylin. D. Expression of TP63 as detected by the pan-p63 monoclonal antibody 4A4 is restricted to the basal epithelia of mammary ducts and lobules. Sections were counterstained with hematoxylin. E. Expression of delta-N-p63 in normal breast detected by delta-N-specific rabbit polyclonal antibody is localized in out layer epithelia of mammary ducts and lobules. The distribution pattern observed by specific delta-N-p63 Ab is identical to that of well known 4A4 pan-p63 antibody. Sections were counterstained with hematoxylin. F. Expression of delta-N-p63 in normal breast was detected with delta-N-p63 specific rabbit polyclonal antibody by immunofluorescence. Positive nuclear signal is restricted to basal epithelia outer layer of luminal epithelial cells. Sections were counterstained with DAPI. G. Staining with TA-p63 specific chicken antibody in normal breast. Expression of TA-p63 is more universal than that of delta-N-p63, not only restricted in basal epithelia, but in inner luminal epithelia. Sections were not counterstained with hematoxylin.

Figure 2.



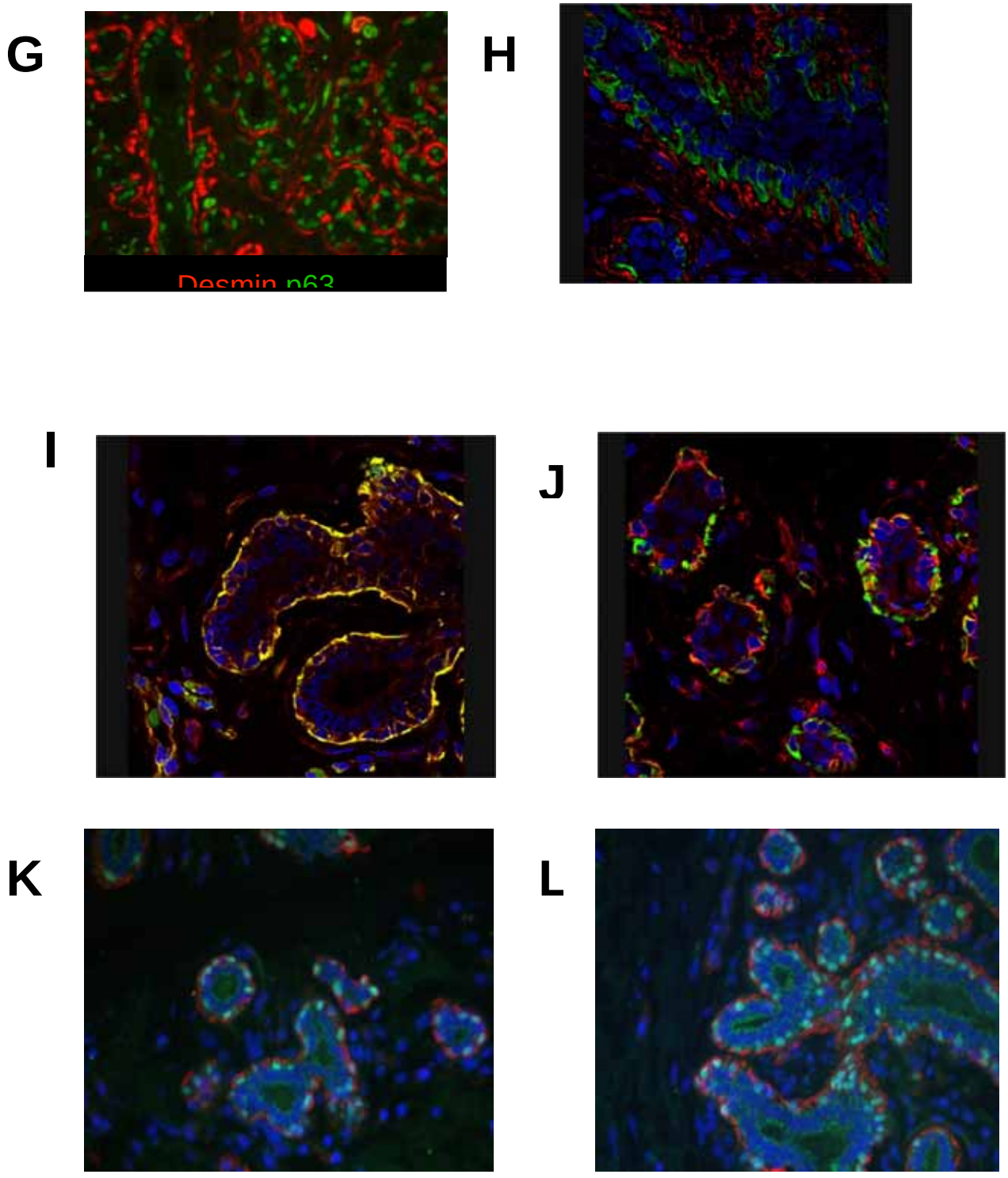
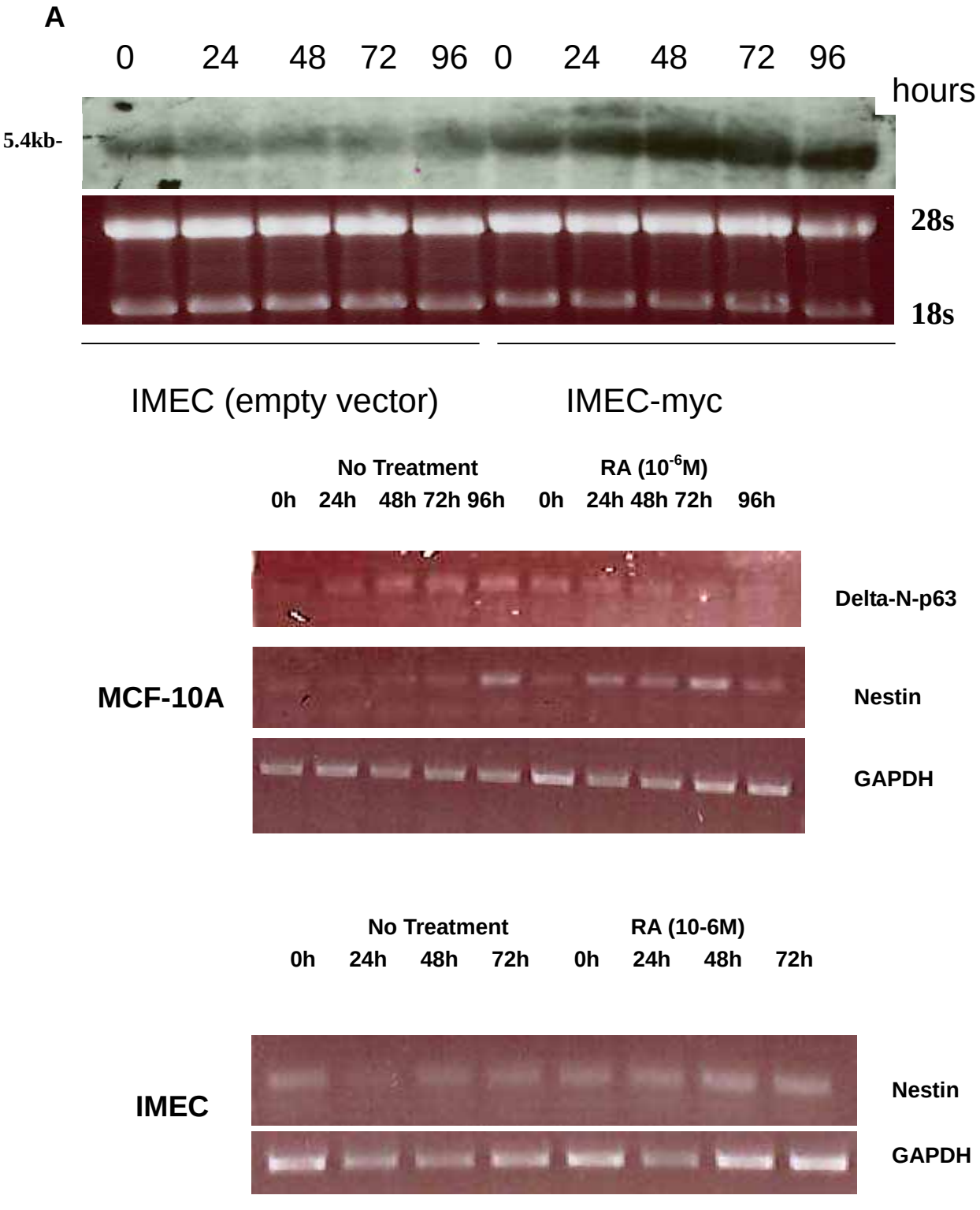


Figure 2. Nestin expression independently co-localizes with basal progenitor markers and with myoepithelial markers. A. Two color

immunofluorescence of FFPE normal human mammary gland indicates that nestin and p63 are co-expressed in a subset of the basal epithelia of mammary ducts. Note (inset) that the red fluorescent signal indicating nestin surrounds the green nuclear signal that indicates p63. B. Similar analyses of mammary lobubles indicates that nestin is co-expressed with delta-N-p63 in the basal epithelia of mammary lobules. Section was counterstained with DAPI. C. D. Two color immunofluorescence indicates co-localization of p63 and CK14. Picture C was staining of delta-N-p63 specific antibody and counterstained with DAPI, picture D was staining of pan-p63 (4A4) antibody. E. Two color immunofluorescence indicates co-localization of CK14 (green) and nestin (red). Note the areas of yellow that indicating the colocalization of nestin and CK14 in the cytoplasm. F. Two color immunofluorescence indicates co-localization of nestin and desmin in the filamentous cells arranged at the periphery of the ducts. G. Two color immunofluorescence indicates that desmin are p63 do not co-localize. Note the regions of desmin staining (red) that are distinct and physically separate from p63 staining (green). H. Two color immunofluorescence indicates that nestin and CK14 do not co-localize in the human mammary gland. Note the distinct red signal of desmin and the distinct green signal of CK14 along with the absence of a yellow signal that would indicate co-localization. Section was counterstained with DAPI. I. Two color immunofluorescence indicates that two nestin primary antibody, staining pattern of goat-anti-human polyclonal one (red) and mouse-anti-human monoclonal one (green) was totally overlapped. Note the yellow signal indicating co-staining and there is no individual red or green signal at all. Section was counterstained with DAPI. J. Two color immunofluorescence indicates that there are some nestin positive basal epithelial cells (red) are not stained with alpha-SMA (green) positively although co-localization of nestin plus alpha-SMA (yellow) could be detected in normal breast duct epithelia. Section was counterstained with DAPI. K. L. Two color immunofluorescence indicates that most delta-N-p63 positive basal epithelial cells (green) are not stained with alpha-SMA positively (red) although a few co-expressed cells could be detected in normal breast ducts and lobules. Note co-localization of delta-N-p63 and alpha-SMA will not produce yellow signal due to different cellular localization of such two protein, namely delta-N-p63 in nuclear and alpha-SMA in cytoplasm. Sections were counterstained with DAPI.

Figure 3.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hours
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Delta-N-p63
MCF-10A
Nestin
GAPDH
No Treatment
RA (10-6M)
0h 24h 48h 72h 0h 24h 48h 72h
IMEC
Nestin
GAPDH

Figure 3: Nestin transcript exists in breast immortalized basal epithelial cell line, IMEC and MCF-10A and Oncogenic transformation of an Immortalized Mammary Epithelial Cell leads to Increased Expression of Nestin. IMECs were stably transfected with empty vector (control) or with expression vector programmed to ectopically express c-myc. Stable transfections were plated at 1,500,000 cells per plate in 10 cm dishes and allowed to attach overnight. Cells were reefed at T0 and RNA was harvested at 0, 24, 48, 72 and 96 hours post feeding. Northern blot was conducted using an ~1kb fragment of an EST constaining a segment of the nestin cDNA. Results (upper panel) indicate that an ~5.4 kb mRNA was detected in all samples. In IMECs transfected with empty vector the nestin mRNA was strongest at T0 and declined to low levels from 24 through 96 hours. By contrast, in the c-myc-transformed IMECs nestin mRNA levels were comparable to T0 for te empty vector controls. However, nestin mRNA was observed to accumulate through 96 hours. The lower panel shows the 28S and 18S ribosomal subunits, stained with ethidium bromide, as loading control. The MCF-10A and IMECs cells were plated at 100,000 cells per well in 6-well plates and refed with RA (10-6 M) at T0 and harvested RNA at 0, 24, 48, 72 and 96 hours post feeding. RNA samples harvested from MCF-10A and IMECs with and without retinoic acid treatment were reverse transcripted to cDNA. In MCF-10A cells, △-N-p63 mRNA level kept increasing from T0 to T96 hours, which is very similar to that in IMECs; and RA treatment could down regulated △-N-p63 mRNA level until T96 hours and such change was not reversible. Interestingly, nestin mRNA level in MCF-10A kept stable until T72 hours, but accumulated at T96 hours eventually. With RA treatment, nestin transcript level was up-regulated from T24 hours to T48 hours, then decreased from T72 to T96 hours again. In IMECs cells, the effect of RA treatment on nestin mRNA level was not so dramatic as in MCF-10A cells, but it could up-regulated nestin transcript at T24 hours, and from T48 to T72 hours, nestin mRNA level decreased again. Different from oncogene transformed IMEC cells, nestin keeps increasing in such malignant breast epithelial cells, RA treatment could reverse such accumulation process at later time course. GAPDH mRNA level was applied as loading control.

Figure 4

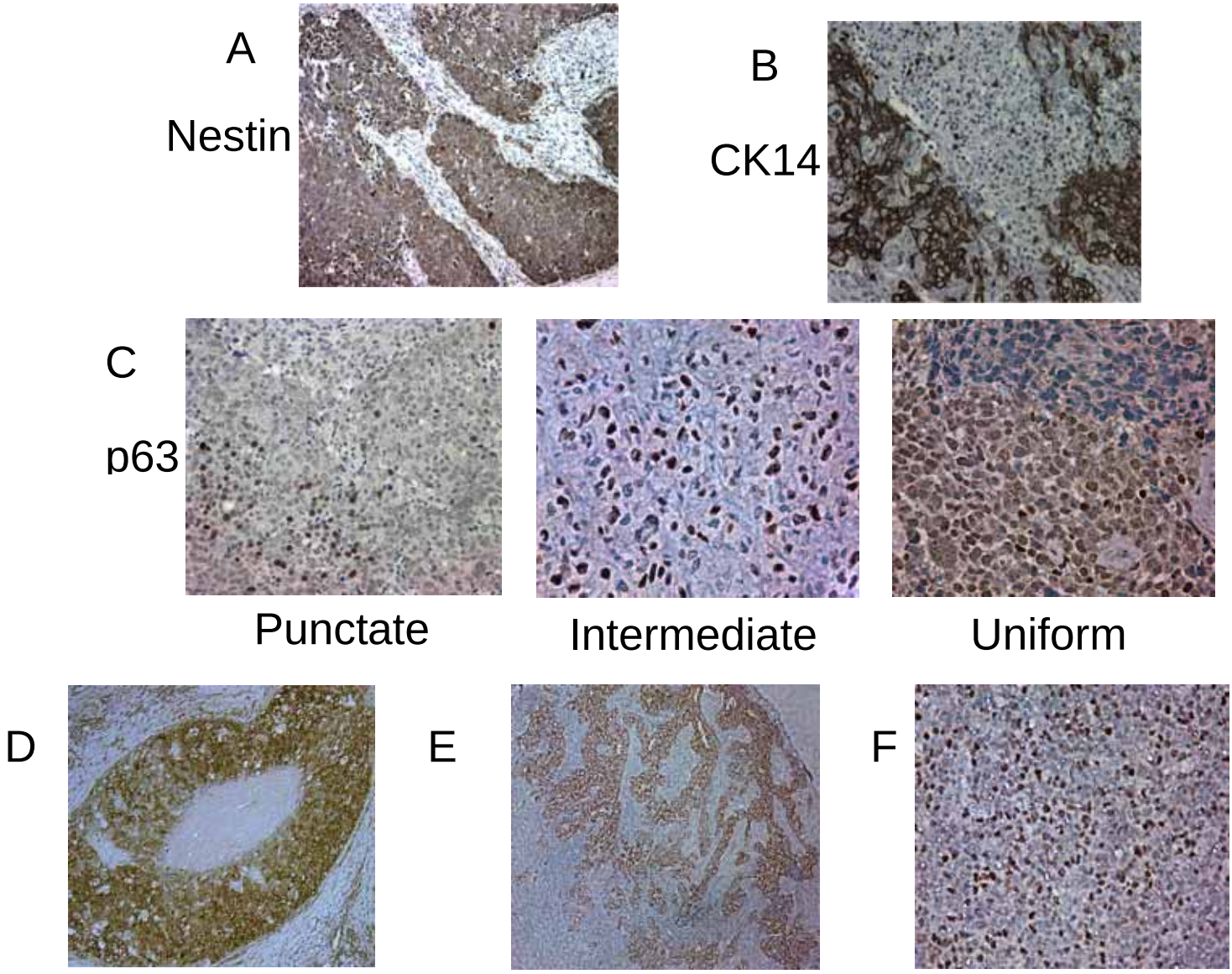

Figure 4: Nestin, CK14 and p63 are selectively expressed in basal epithelial breast tumors and BRCA-1 associated tutors. Tumors that were previously known to lack expression of Erα, PR and Her2 were prescreened for expression of CK5/6 to confirm the basal epithelial phenotype. A. Representative immunohistochemical staining of FFPE breast tumors with a basal phenotype indicates robust expression of nestin. Anti-nestin IHC was performed as described in Methods. B. Representative immunohistochemical staining of FFPE breast tumors with a basal phenotype indicates robust expression of CK14. Anti-CK14 IHC was performed as described in Methods. C. Expression of p63 was detected in

8 of 16 breast tumors with as basal epithelial phenotype. Expression patterns ranged from punctate (left panel), to intermediate (center panel) to uniform (right panel). BRCA-1 associated breast tumors express robust levels of nestin, CK14 and p63. Breast tumors with confirmed mutations in BRCA-1 were identified from the tissue and tumor bank at Fox Chase Cancer Center. D. Immunohischemical analyses reveal robust detection of nestin in BRCA-1 associated tumors. E. Immunohischemical analyses reveal robust detection of ck14 in BRCA-1 associated tumors. F. Immunohischemical analyses reveal intermediate detection of p63 in BRCA-1 associated tumors.

Table 1: Expression of nestin, CK14 and p63 in breast cancer

Sub-type	Markers	Nestin	CK14	p63
Basal	ERα-/PR-/Her2-	14/16 (2.6E-9)	16/16 (3.77E-11)	8/16 (6.77E-5)
Her2-associated	ERα-/PR-/Her2+ (FISH)	0/16	0/16	0/16
Luminal	ERα+/PR+	0/16	0/16	0/16

Table 1: Summary of expression of nestin, CK14 and p63 in three distinct subtypes of breast tumors. Results indicate that nestin, CK14 and p63 are selectively expressed in tumors with a basal epithelial phenotype. P values (shown in parentheses) reflect the likelihood that the distribution of each of these markers was observed by chance.

Bullets of accomplishments:

1. Generation of delta-N-p63 and TA-p63 specific primary antibody.
2. Immunohistocheminstry analyses of nestin, delta-N-p63, CK14, CK5/6, alpha-SMA expression in normal breast to confirm their localization in basal epithelia or myoepithelia.
3. Immunohistochemistry analyses of nestin, delta-N-p63, alpha-SMA expression in breast cancer, including ER, PR positive; ER, PR negative, Her2 positive, and triple negative (ER, PR, Her2 negative) subtype.
4. Immunohistochemistry analyses of nestin, delta-N-p63 expression in BRCA-1 associated breast cancer.
5. Localization of two immortalized breast basal epithelia cell lines, IMEC and MCF-10A as model for further investigation of nestin biological function and its correlation with delta-N-p63 in regulation of progenitor self-renewal or differentiation.
6. IMCE transformed with stable c-myc transfection as a cellular model for further investigation of biological behavior of triple negative breast cancer, which is abundant in progenitor marker delta-N-p63 and promising candidate, nestin.

Reportable Outcome

One manuscript has been submitted to Cancer Research and is pending for second review.

Hua Li, Pratima Cherukuri, Alissa Pho, Victoria Cowling, Michael Cole, Andrew K Godwin, Wendy Wells and James Direnzo. Nestin is expressed in putative mammary progenitors and is a selective marker of basal epithelial breast tumors. Cancer Research.

Conclusions

In this annual report we report that nestin is expressed in two morphologically distinct cell types within the basal epithelial layer of the mammary gland. Two-color immunofluorescence indicates a cell type in which nestin is co-expressed with cytokeratin 14 and p63 and a second that is positive for desmin. The result of RT-PCR has confirmed nestin transcript exists in two immortalized breast basal epithelial cell line, IMECs and MCF-10A. Oncogenic transformation of an Immortalized Mammary Epithelial Cell (IMEC) line with features of SRBPs leads to increased and sustained expression of nestin. Taken together, these observations suggested that nestin might be expressed in breast cancers with a basal epithelial phenotype. Immunohistochemical analysis indicates that nestin is robustly expressed in basal epithelial breast tumors (defined as triple-negative for the estrogen receptor-α (ER) the progesterone receptor (PR) and Her2 and positive for cytokeratin 5/6 and undetectable in breast tumors representing other molecular classifications. We also present data indicating that nestin is strongly expressed in BRCA1- associated breast tumors which is consistent with the finding that BRCA1-associated tumors cluster with the basal epithelial sub-type. Further analysis of the triple-negative tumors indicates high levels of cytokeratin 14, punctate expression of p63 and undetectable levels of desmin, suggesting that these tumors arose from components of the basal epithelia that express nestin, CK14 and p63. These studies indicate that nestin expression identifies the basal epithelial phenotype and may be correlated with poor prognosis. They also suggest that the highly aggressive and poorly differentiated basal breast cancer subtype displays many of the features of normal mammary SRBPs.

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